REMARKS

Claims 1-10, 12-14, 26-30, 33 and 34 were pending prior to entering the amendments.

Claim 1 is amended to correct a typographic error.

No new matter is added in any of the amendments. The Examiner is requested to enter the amendments and re-consider the application.

35 U.S.C. §102(a) Rejection

4. Claims 1, 2, 4, 8, 9 and 12-14 are rejected under 35 U.S.C. §102(a) as allegedly being anticipated by Krebs et al., "Detection of fast light activated H+ release and M intermediate formation from proteorhodopsin", BMC Physiology, Vol. 2 pp. 5-12 (8 pages) (04/2002). The rejection is respectfully traversed.

Krebs et al. used SDS/gel electrophoresis to check the purity of PR; the gel was not an optical information carrier. **The protein sample was denatured by boiling in SDS as required by the SDS-PAGE procedures, before it was loaded on gel.** The denatured proteins were separated by molecular weight during electrophoresis. This is evidenced by Laemmli, *Nature*, 227:680-685, 1970, which describes the polyacrylamide gel electrophoresis procedures used in Krebs et al (see page 7 or Krebs). At page 681, fine prints of *Gel electrophoresis*, Laemmli describes that the samples contained 2% SDS, 5% 2-mercaptoethanol, and "the proteins were completely dissociated by immersing the samples for 1-5 min in boiling water. A copy of Laemmli is attached herewith. This is further confirmed by Krebs et al, as Krebs et al describe the gel loading solution was boiled for several minutes (See Krebs at page 3, left column, first paragraph).

In Krebs et al, although the purified PR was immobilized in the polyacrylamide gel after electrophoresis, PR was denatured and was not capable of producing a functional photocyle when exposed to light of excitation wavelength.

Other than the denatured PR that was immobilized in the polyacrylamide gel after electrophoresis, Krebs et al. only describe a basic research that examines the physical properties of PR in a solution phase; PR was not fixed to a solid and was not in an

immobilized format. Krebs et al. did flash photolysis with reconstituted PR in a solution phase, in which the PR is detergent-solubilized, cellular membrane-free and in a monomer or oligomer form. Krebs et al. extract PR from membrane with a detergent β-octyl-D-glucoside. The column-purified PR was reconstituted into mixed micelles containing 1,2-diheptanoyl-SN-glycero-3-phosphocholine (DHPC), a phospholipid.

At page 6, last paragraph, Krebs et al. state that "The requirement for pR to be in lipid to show fast H^+ release and M formation stems either from a protein/lipid interaction needed to establish a stable, active tertiary structure, or from the need for the phosphate group in DHPC to act as a proton release group." In the second paragraph of Conclusion at page 7, Krebs et al state "The necessity of reconstituting pR with some lipid before it is capable of photocycling shows that the presence of lipids facilitates pR in assuming its fully active structure." Krebs et al state that pR needs to be reconstituted with lipids before being capable of photocycling/M state formation, thus Krebs et al. teach away from the present invention of an optical information carrier comprising immobilized PR, which is detergent-solubilized, cellular membrane-free, and in a monomer or oligomer form, said proteorhodopsin is capable of producing a photocyle when exposed to light of excitation wavelength.

According to Krebs et al, there is no reasonable expectation of success by immobiling detergent-solubilized, cellular membrane-free, monomer/oligomer form of PR onto a solid to produce an optical information carrier. However, in the present application, Applicants have provided a working example of optical data storage using proteorhodopsin-PVA film, where the PR is detergent-solubilized, cellular membrane-free, and in a monomer or oligomer form. (See application, Example 9).

Therefore, the 102(a) or 103(a) rejection over Krebs should be withdrawn.

35 U.S.C. §103(a) Rejection

5. Claims 1, 2, 4, 8, 9 and 12-14 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Krebs et al., in view of Friedrich et al. "Proteorhodopsin is a light driven proton pump with variable vectorality", J. Mol. Biol. Vol. 321(5) pp. 821-838 (8/2002). The rejection is respectfully traversed.

As discussed above, Krebs et al do not teach or suggest immobilized a functional, non-denatured PR that is capable to produce a functional photocycle.

Friedrich et al. do not teach immobilized PR that is detergent solubilized, in a monomer or monoer or oligomer form.

The Examiner states that Friedrich et al. teaches the measurement of absorption spectra with PR embedded in 1 mm thick acrylamide gels (page 835, left column). Although Friedrich et al. disclose the purification of proteorhodopsin (PR), **all of the spectroscopic data cited by the Examiner were obtained using PR reconstituted in phospholipid membrane vesicles**, and not using purified PR. This is acknowledged by the Examiner at page 5 of the Final Office Action.

Therefore, the combination of Krebs et al., in view of Friedrich et al. does not produce immobilized PR that is detergent solubilized, cellular membrane-free, in a monomer or oligomer form, and is functional as an optical information carrier. **The detergent-solubilized PR in the form of monomer/oligomer has unexpected advantages over phospholipid vesicle-containing PR in that the former does not cause light scattering, thus providing a good signal-to-noise ratio** (see Application at page 3, lines 25-29).

6. Claims 1-10, 12-14, 26-30 and 33-34 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Krebs et al., in view of Friedrich et al., further in view of Hampp et al. '279 and/or Wu et al. "Bacteriorhodopsin encapsulated in transparent solgel glass: A new biomaterial", Chem. Mater. Vol. 5 pp. 115-120 (1993).

As discussed above, the combination of Krebs et al., in view of Friedrich et al. does not produce immobilized PR that is detergent solubilized, cellular membrane-free, in a monomer or oligomer form, and is functional as an optical information carrier (Claims 1, 29 and their dependent claims).

Claims 26 and 28 are directed to a method of optically storing information on a material or a method of producing a three-dimensional optical image for information storage, comprising directing onto only a selected portion of a material containing immobilized proteorhodopsin light of a first spectral range representing optical information to be stored; and exposing the selected portion of the material containing

immobilized proteorhodopsin to switch the proteorhodopsin from its basal state to its M state. Krebs et al. and Friedrich et al. do not teach or suggest directing light onto only a selected portion.

The addition of Hampp et al., which only disclose bacteriorhodopsin (BR), does not cure the deficiency of Krebs et al. or Friedrich et al. Hampp et al. use native purple membrane patches, which are micrometer sized patches containing a 2D crystal of lipids and BR proteins. Hampp et al. do not teach or suggest PR, let alone detergent-solubilized PR in the form of monomer/oligomer.

Wu et al. only disclose bacteriorhodopsin encapsulated in sol-gel. Wu et al. do not mention proteorhodopsin. Therefore, the addition of Wu et al does not cure the deficiency of other cited references.

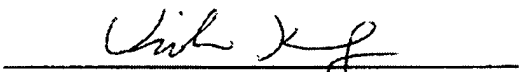
Therefore, the 103(a) rejection of Claims 1-10, 12-14, 26-30 and 33-34 over Krebs, Friedrich et al., Hampp, and Wu should be withdrawn.

CONCLUSION

Applicants believe that the application is now in good and proper condition for allowance. Early notification of allowance is earnestly solicited.

Respectfully submitted,

Date: January 7, 2008



Viola T. Kung, Ph.D. (Reg. No. 41,131)

Enclosure: Laemmli, *Nature*, 227:680-685, (1970)

HOWREY LLP
2941 Fairview Park Drive
Box 7
Falls Church, VA 22042
Tel: 650-798-3570

demonstrated a shielding effect of the ribosome on nascent proteins; ribosome-bound nascent chains 30–35 residues long were found to be protected from external proteolytic attack. Protamine, 31–32 residues long, may therefore be protected from attack by a putative methionine-removing enzyme until the chain is completed and released into the cytoplasm. Other contributory circumstances may be the difficulty of cleavage of the Met-Pro peptide bond; in general, X–Pro bonds are resistant to many proteases with the possible exception of imidodipeptidase[30]; but this enzyme is specific only for dipeptides. Also, as shown by Ling and Dixon[28], at the stage of differentiation at which protamine synthesis is maximal, there has been a considerable reduction of the spermatid cytoplasm and ribosome content. The levels of cytoplasmic enzymes, including the methionine-removing enzyme, may therefore become limiting at this stage.

The involvement of methionine in the initiation of protein synthesis in eukaryotic cells is not yet firmly established although the presence of a formylatable species of Met-tRNA with the properties of an initiator tRNA in mouse ascites cells[31,32] is certainly consistent with such a role. Our observations of methionine involvement in the synthesis of the very unusual, sperm-specific polypeptide, protamine, suggest that such a mechanism may be of widespread importance in eukaryotes, and in the special circumstances of protamine biosynthesis, where removal of the N-terminal methionyl residue may become limiting, the transient incorporation of this amino-acid is readily observed.

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Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4

by
U. K. LAEMMLI
MRC Laboratory of Molecular Biology, Hills Road, Cambridge

Using an improved method of gel electrophoresis, many hitherto unknown proteins have been found in bacteriophage T4 and some of these have been identified with specific gene products. Four major components of the head are cleaved during the process of assembly, apparently after the precursor proteins have assembled into some large intermediate structure.

Bacteriophages of the T-even type are complex structures containing many different proteins and specified by many genes. Using an improved technique of electrophoretic separation I have found that the phage particle contains at least twenty-eight components, eleven of which are in the head. In the course of identifying the genes specifying these proteins I discovered that four major components of the head, the product of gene 22, 23, 24 and a protein called IP of unknown genetic origin are cleaved during the process of assembly. The head of bacteriophage T4 is therefore no longer a self-assembly system in the narrow sense, because the bonding properties of the various components become altered during the assembly process.

The product of gene 23 is the principal protein component of the head of bacteriophage T4 (refs. 1–3). Two minor components of unknown genetic origin have also been found in capsids[2,3]. Besides the product of gene 23, the products of genes 20, 21, 22, 24, 31, 40 and 66 are required to determine the size and shape of the head-shell (refs. 4 and 5 and unpublished work of F. A. Eiserling, E. P. Geiduschek, R. H. Epstein and E. J. Metter). To several of these genes shape-specifying functions have been tentatively assigned[4]. Gene 22 is associated with the diameter selecting (initiation) process of head formation, gene 66 with the elongation of the particle and genes 20 and 40 with the formation of the hemispherical cap. Gene 31 somehow modifies or activates the major subunit for ordered assembly[4]. Ten more proteins, the products of genes 2, 4, 13, 14, 16, 17, 49, 50, 64 and 65, are thought to control later steps in head formation[7].

Structural Components of the Phage

Many phage proteins can be separated with our improved method of disk-electrophoresis in sodium dodecyl sulphate (SDS). This system, to be described in detail elsewhere (U. K. L. and J. V. Maizel), combines the high resolution power of disk-electrophoresis[6] with the capability of SDS to break down proteins into their individual polypeptide chains[8]. The proteins are also separated according to their molecular weight as was first reported for a continuous system[10]. All the proteins the genetic

Fig. 1. Autoradiogram of ^{14}C-labelled T4 phage proteins separated in acrylamide gels. ^{14}C-amino-acid-labelled preparations were analysed in 10 per cent acrylamide gels containing SDS. *a*, Wild type lysate; *b*, purified heads; *c*, purified phage particles; *d*, purified "ghosted" phage particles; *e*, supernatant of "ghosted" phage particles; *f*, "early labelled" phage particles. The prefix P is used to designate the protein of a particular gene: for example, P20 stands for the product of gene 20; the asterisk indicates that this protein is derived from a large precursor protein and has become modified during head assembly.

^{14}C-labelled lysates. Ten ml. cultures of *Escherichia coli* B^{b} (the restrictive host for phage carrying amber mutations) in 'M9' medium[10] grown at 37° C to 2×10^{8} cells/ml. were infected with the various phages at a multiplicity of five and superinfected at 8 min with the same phage and the same multiplicity to ensure lysis inhibition. Two μCi of ^{14}C-amino-acid mixture ('CFB 104', Radiochemical Centre, Amersham) with a specific activity of 45 mCi/m atom was added to each culture 13 min after the first infection. Three ml. of 3 per cent 'casamino-acids' mixture (Difco) was added to each sample at 30 min, and the infected cells were concentrated by low speed centrifugation 35 min following infection. The pellets were drained and directly resuspended in "final sample" buffer (see gel electrophoresis).

^{14}C-labelled phage and head particles. Ten ml. cultures were grown and infected as described above (^{14}C-labelled lysates). The double mutant (B255–E18) in genes 10 and 18 was used for production of tailless heads. Ten μCi of the ^{14}C-amino-acid mixture was added 13 min after the first infection to each culture. The infected cells were concentrated by a low speed centrifugation 35 min after infection and the pellet was resuspended in 1 ml. neutral phosphate buffer containing 10^{-2} M $MgSO_4$, 20 μg deoxyribonuclease and a drop of chloroform. The pellet was resuspended by repeated pipetting, and incubated for 15–30 min at room temperature before layering on a CsCl step gradient[4]. The latter was prepared in tubes for a Spinco 'SW50' rotor, with 0·8 ml. layers, and the following densities starting at the bottom of the tube: 1·55, 1·46, 1·38 and 1·20 g/cm^3. Furthermore, a 10 per cent sucrose solution (in neutral phosphate buffer and 10^{-2} M $MgSO_4$) was layered on the last CsCl step to prevent precipitation of soluble proteins at the CsCl interface. Centrifugation was for 1 h at 40,000 r.p.m. The phage and heads, which form a sharp visible band two-thirds down the tube, were collected through the bottom of the tube and dialysed against water. The band containing the heads was always viscous, indicating that the heads lost their DNA in CsCl although the DNA was still confined within the band. Occasionally the heads lost their DNA before centrifugation particularly in more concentrated lysates, which had to be treated with deoxyribonuclease for a much longer time. "Early labelled" phages were prepared identically, but the label was added 1 min and chased 6 min after infection by the addition of 3 ml. of 3 per cent 'casamino-acids'.

"Ghosted" phage particles and supernatant of "ghosted" phage particles. Crystalline NaCl was added to ^{14}C-labelled purified phage particles in water to a final concentration of 5 M. The preparation was then repeatedly frozen in a solid CO_2-acetone bath and thawed in warm water. This procedure releases the DNA and its internal proteins from the phage head. The sample was dialysed against neutral phosphate buffer containing 10^{-2} M $MgSO_4$ and the DNA digested by the addition of a small amount of crystalline deoxyribonuclease. The ghost was finally separated from the soluble proteins (internal proteins) by centrifugation and layered on a step gradient identical to that already described. Centrifugation was at 35,000 r.p.m. for 1 h. The top of the gradient containing the internal proteins was collected with a pipette and the ghosts, which band at an approximate density of 1·3 g/cm^3, were collected through the bottom of the tube. SDS at a final concentration of 2 per cent was added to both samples and the samples were dialysed against water containing 2 per cent SDS.

Gel electrophoresis. Gels containing 3 per cent (stacking gel), 8·0 per cent or 10 per cent acrylamide were prepared from a stock solution of 30 per cent by weight of acrylamide and 0·8 per cent by weight of *N,N'-bis*-methylene acrylamide. The final concentrations in the separation gel were as follows: 0·375 M Tris-HCl (*p*H 8·8) and 0·1 per cent SDS. The gels were polymerized chemically by the addition of 0·025 per cent by volume of tetramethylethylenediamine (TEMED) and ammonium persulphate. Ten cm gels were prepared in glass tubes of a total length of 15 cm and with an inside diameter of 6 mm. The stacking gels of 3 per cent acrylamide and a length of 1 cm contained 0·125 M Tris-HCl (*p*H 6·8) and 0·1 per cent SDS and were polymerized chemically in the same way as for the separating gel. The electrode buffer (*p*H 8·3) contained 0·025 M Tris and 0·192 M glycine and 0·1 per cent SDS. The samples (0·2–0·3 ml.) contained the final concentrations ("final sample buffer"): 0·0625 M Tris–HCl (*p*H 6·8), 2 per cent SDS, 10 per cent glycerol, 5 per cent 2-mercaptoethanol and 0·001 per cent bromophenol blue as the dye. The proteins were completely dissociated by immersing the samples for 1·5 min in boiling water[9]. Electrophoresis was carried out with a current of 3 mA per gel until the bromophenol blue marker reached the bottom of the gel (about 7 h). The proteins were fixed in the gel with 50 per cent trichloroacetic acid (TCA) overnight, stained for 1 h at 37° C with a 0·1 per cent Coomassie brilliant blue solution made up freshly in 50 per cent TCA. The gels were diffusion-destained by repeated washing in 7 per cent acetic acid. Autoradiograms of gels were prepared by a modified version (U. K. L. and J. V. Maizel, unpublished) of Fairbanks *et al.*[8] (autoradiograms are shown in Figs. 1–7).

origin of which was determined are labelled in Fig. 1, and their molecular weights are listed in Table 1. At least 28 bands can be distinguished in the autoradiogram of radioactively labelled, purified phage particles (Fig. 1*c*). The 28 proteins found in dissociated phage particles do not include proteins with molecular weights less than about 15,000. These proteins are not sieved in gels of 10 per cent acrylamide (unpublished results of U. K. L. and J. V. Maizel) and migrate with the marker dye. In gels of higher acrylamide concentration another three low molecular weight proteins have been separated (results not shown). The largest protein in the phage has an approximate molecular weight of at least 120,000 and no label stays at the top of the gel, indicating complete dissociation of the particles by the method used.

Eleven of these 28 proteins are found in the purified head preparation (Fig. 1*b*). The remaining 17 proteins absent from the head but present in the whole phage pattern are presumably structural proteins of the tail and tail fibres. The complete absence of these proteins from the head gel pattern indicates the high degree of purity of the preparation. The classification of the proteins into tail and head components may not be valid for proteins making up the head to tail junction.

Only two minor proteins besides the major components P23 were found by others[1,2] in phage capsids purified in the same way but fractionated in urea gels. The larger number of proteins found in SDS gels is to be expected, for at least 46 genes are known to affect T4 morphogenesis[4,7] although the proteins of these genes may not all be incorporated into the particle.

The gel pattern of a total lysate of wild type infected cells radioactively labelled at late times is presented in Fig. 1*a*. Note that most of the resolved proteins that are synthesized late in infection are structural phage components. A few rather intense bands (X_1 and X_2), however, are completely missing in the phage particles, also demonstrating that the separation of the phage particles from the soluble proteins is complete.

When phages are subjected to osmotic shock a number of proteins are released[11]. The principal one is 1P*, an internal protein (Fig. 1). It is present in complete phage particles, is extracted from the phage particles by freezing and thawing in high salt concentrations and is quantitatively recovered in the supernatant. The supernatant fraction also contains many minor components which are found both in phages as well as phage ghosts, indicating that the

Table 1. MOLECULAR WEIGHTS OF PHAGE PROTEINS DETERMINED BY COMPARING THEIR MOBILITY IN SDS GELS WITH THOSE OF MARKER PROTEINS WITH KNOWN MOLECULAR WEIGHTS[10,12]

Gene product	Observed value in SDS gels	Published value
P18	69,000	50,000[3]
P20	63,000	
P23	56,000	
P23*	46,500	46,000[11]
P24	45,000	
P24*	43,500	
P22	31,000	
1P	23,500	
1P*	21,000	
P19	18,000	

The following marker proteins were used: serum albumin (68,000), γ-globulin, heavy chain (50,000), ovalbumin (43,000), γ-globulin, light chain (23,500) and TMV (17,000), to calibrate 10 per cent acrylamide gels. In this improved gel system the distances of migration of the various marker proteins relative to the distance of migration of bromophenol blue are also a linear function of the logarithm of the molecular weight of the marker proteins, as has been described[10,12]. Radioactively labelled phage proteins were mixed with unlabelled marker proteins before electrophoresis; the distance of migration of the phage proteins was determined from the autoradiogram and those of the marker proteins from the stained gel. It is assumed that the phage proteins also separate in SDS gels solely according to their molecular weight.

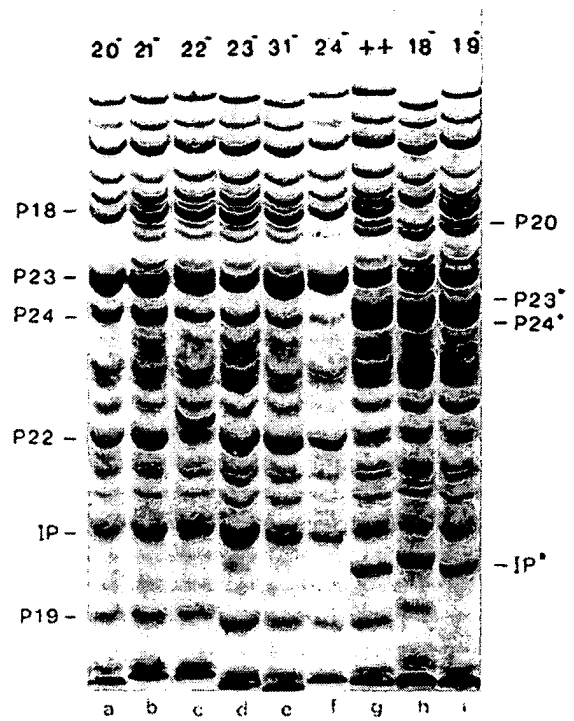

Fig. 2. Identification of gene products on 10 per cent acrylamide gels. The lysates were prepared as described in Fig. 1 and analysed on 10 per cent acrylamide gels. *a*, Gene 20-defective lysate, mutant N50; *b*, gene 21-defective lysate, mutant N90; *c*, gene 22-defective lysate mutant B270; *d*, gene 23-defective lysate, mutant H11; *e*, gene 31-defective lysate, mutant N54; *f*, gene 24-defective lysate, mutant N65; *g*, lysate from wild type infected cell; *h*, gene 18-defective lysate, mutant E18; *i*, gene 19-defective lysate, mutant E1137. Mutants N90 (gene 21), E18 (gene 18) and E1137 (gene 19) carried second mutations in gene 10 (mutant B255). The following amber fragments may be detected. A rather intense band just below P23 is consistently seen in 18-defective lysates, and is presumed to be the amber fragment of mutant E18 in gene 18. Furthermore, the gel pattern of defective lysates of all double mutants in genes 18–10, 21–10, and 19–10 possess a band just above P23 which is probably the amber fragment of mutant B255 in gene 10. It is striking that the amber fragment of mutant B270 gene 22 behaves anomalously in the gel: it migrates more slowly than the wild type product, although SDS gels are known to separate on the basis of molecular weights. This anomalous behaviour of certain proteins will be discussed elsewhere (U. K. L. and J. V. Maizel).

separation of the released proteins and the ghosted particles by centrifugation was not complete. Some of these proteins, however, are extracted into the supernatant quantitatively by the freezing and thawing procedure.

Only the protein IP*, and perhaps some low molecular weight proteins which migrate with the marker dye, are structural phage components synthesized at early times (early proteins). This can be seen in the gel pattern of purified phage labelled at early times only (Fig. 1*f*). IP* is also labelled at late times. All the other structural phage components are synthesized only late in infection. Some of the principal late proteins show up on the autoradiogram, probably because of residual incorporation of radioactive amino-acids following the chase with unlabelled amino-acids.

Identification of Gene Products

The products of genes 18, 19, 20, 22, 23 and 24 were identified by comparing the gel pattern of extracts of cells infected with wild type phage with those infected with amber mutants in various genes. The identification of the tail and tail fibre proteins will be described later (J. King and U. K. L.). Amber mutations produce only fragments of the protein chain of the mutant gene on infection of restrictive bacteria[1]. These fragments migrate differently in the gel from the complete proteins. The molecular weights of the proteins identified are listed in Table 1. Fig. 2 is the autoradiogram from dried and sliced gels for various mutants. The product of gene 20 is identified by its absence in the gel pattern of a 20-defective* lysate (Figs. 2 and 3*a*), and the product of gene 22 by its absence in the gel pattern of a 22-defective lysate (Figs. 2 and 3*c*). Note the amber fragment of mutant B270 in gene 22. This fragment was also identified by Hosoda and Levinthal[13] in urea gels (see legend to Fig. 2).

The product of gene 23 is easily identified by its absence in the gel pattern of a 23-defective lysate (Fig. 2*d*). It can also be seen that P23 overlaps with two minor tail components. If the 23-defective lysate is analysed on gels of lower acrylamide concentration another important observation is made. A band, P23*, which is detected in variable amounts in the other head defective lysates, is completely missing in the 23-defective lysate (Fig. 3*d*). This band, P23*, overlaps with P24 in Fig. 2, but is better separated from P24 in less concentrated gels (Fig. 3). As with the product P23, the product of P24 was identified by its absence in the gel pattern of a 24-defective lysate (Figs. 2*f* and 3*e*).

So far, no missing bands have been found in the gel patterns of 31 or 21-defective lysates (Fig. 2*b* and *e*), but analysis of the 21-defective lysate on gels of lower acrylamide concentration (Fig. 3*b*), which resolves higher molecular weight proteins better, clearly shows that a band is missing. This protein, however, is the product of gene 10, a baseplate gene. The mutant N90 in gene 21 in fact carries a second mutation in gene 10 (mutant B255).

In comparing the gel pattern of the head-defective lysates (Fig. 2*a–f*) with that of wild type (Fig. 2*g*), further important differences are observed, which shed light on the precursor–product relationship of the head components.

The principal fraction of the gene 23 product has a molecular weight of 56,000 in all the head-defective lysates, but in wild type or tail-defective lysates it appears at the position of P23*, with a molecular weight of 46,500. Small but significant amounts of P23* are also observed in head-defective lysates (Fig. 3*a–f*). In lysates prepared identically about 20 per cent of the total P23 is converted to P23* in the 20-defective lysate, 10 per cent in 21 and 2–3 per cent in 22, 24 and 31-defective lysates, as determined from densitometer tracing of the autoradiographs.

The bands P22 and IP are both absent or considerably less intense in the wild type gel pattern (each overlaps with two other proteins). A new band, IP*, is seen at the bottom of the gel, which is completely missing in the head defective lysates (Fig. 2*a–f*).

The band P24, which is found in all head-defective lysates is missing in the wild type pattern, but a new band, P24*, which migrates slightly faster is observed. This is difficult to visualize in Fig. 2, but will become evident in Fig. 6.

Also included in Fig. 2 is the gel pattern of two tail defective lysates. The product of gene 18 (mol. wt 69,000), the principal protein of the tail sheath[12], is identified by its absence in an 18-defective lysate (Fig. 2*h*) and the product of gene 19 (mol. wt 18,000) by its absence in a 19-defective lysate (Fig. 2*i*). P19 is thought to be the chief component of the tail tube[14]. This demonstrates that the differences in the head proteins are not related to tail attachment, for the gel patterns of the tail-defective lysates are identical with that of wild type.

Evidence will be presented that the proteins P23, P22, P24 and IP are cleaved in wild type infected cells and are precursors to proteins P23*, P24* and IP* found in the final head structure. (The cleavage product of P22 was not detected.) This precursor–product conversion is strongly inhibited by mutations in genes 20, 21, 22, 23, 24 and 31. Two important conclusions can be drawn: (*a*) the aberrant head-related structures—single and

* Henceforth, lysates of cells infected at restrictive conditions with amber mutants in various genes will be referred to as, for instance, a "21-defective lysate", where the amber mutant used was in gene 21.

multi-layered polyheads[4,5,15], τ-particles[4,5,16] and lumps[6] known to be produced in these mutant infected cells—chiefly consist of the precursor protein P23; (*b*) because P22 is not cleaved in these mutant infected cells, but is required for polyhead and τ-particle formation, as has been established by genetic means[5], it is strongly suggested that P22 is incorporated into these structures as such.

Kinetics of the Cleavage Reactions

The following experiments were designed to study the precursor relationship of the proteins P23, P24 and IP with P23*, P24* and IP*, respectively. In this experiment the infected cells were pulse-labelled with radioactive amino-acids for a short period (1 min) and the modification of the various proteins was then followed by analysis of the samples taken at intervals in SDS gels. The results of autoradiography of the dried gels are presented in Fig. 4.

Cleavage of P23. It is readily seen in Fig. 4 that most of the 23 protein is at the position of P23 immediately following the pulse of the radioactive amino-acids. It then rapidly disappears, and a new band, P23*, appears simultaneously. That P23 is cleaved and gives rise to P23* is suggested by the fact that both are principal components and this is reinforced by the absence of P23 and P23* from the gel patterns of a 23 defective lysate (Fig. 3*d*). The kinetics of the cleavage reaction P23→P23* are plotted in Fig. 5*a*. Cleavage is very rapid: about 50 per cent of the precursor is cleaved within the first 2 min following chase of the label. P23* appears at about the same rate, in a satisfactorily correlated way. The total labelled protein in P23 and P23* is also plotted in Fig. 5*a*. The total label increases during the first minute, which reflects the completion time of the chase of the labelled amino-acid, but finally the total falls off by 25–30 per cent. This final decrease of the total labelled protein can be nicely explained, for a cleavage from about 56,000 to 46,500 corresponds to a loss of about 20 per cent by weight of protein.

Cleavage of P22 and IP. In the pulse-chase experiment of Fig. 4, two other protein bands, P22 and IP, disappear with time. The disappearance of P22 in wild type infected cells has also been observed by M. Showe, personal communication.) A new band, IP*, appears at the bottom of the gel pattern. The kinetics of these cleavage reactions are plotted in Fig. 5*b*. P22 disappears with approximately the same initial rate as P23: about 50 per cent is cleaved 2–3 min following chase of the labelled amino-acids. I have not found a band in the gel pattern which may be derived from P22. Hosoda and Levinthal[12] reported indirect evidence that P22 is a structural phage component, but they considered the possibility that P22 might become altered during head formation.

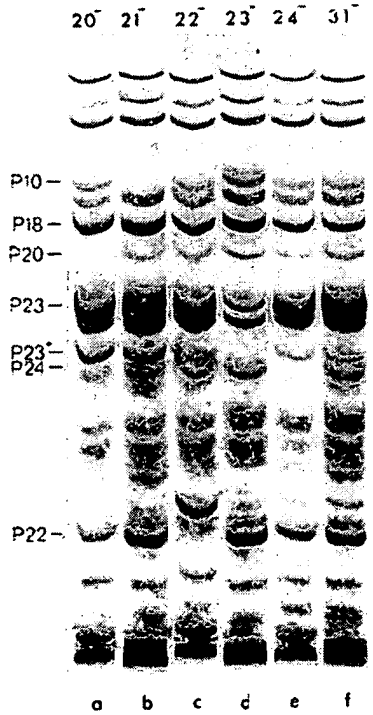

Fig. 3. Identification of gene products on 8 per cent acrylamide gels. ^{14}C-labelled lysates were prepared as described (Fig. 1) and analysed on 8 per cent acrylamide gels. *a*, Gene 20-defective lysate, mutant N50; *b*, gene 21-defective lysate, mutant N90; *c*, gene 22-defective lysate, mutant B270; *d*, gene 23-defective lysate, mutant H11; *e*, gene 24-defective lysate, mutant N65; *f*, gene 31-defective lysate, mutant N64.

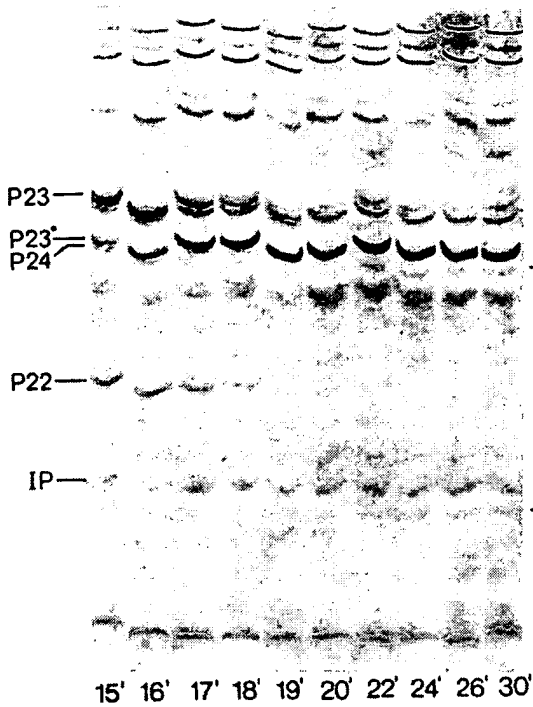

Fig. 4. Cleavage of products of genes 22, 23, 24 and protein IP (10 per cent acrylamide gels). A 10 ml. culture grown at 37° C was infected with a double mutant defective in genes 10 and 18 (mutant B255 and E18) as described (Fig. 1). The radioactive amino-acid mixture (10 μCi) was added 14 min after the first infection, and chased 1 min later with an excess of unlabelled amino-acids (final concentration 1 per cent). The chase of the label was verified by measuring the counts in the total TCA precipitable proteins. One ml. samples were prepared at intervals after the chase and immediately frozen in a solid CO_2-acetone bath. SDS was added after thawing to a final concentration of 2 per cent and the samples were carefully dialysed into 2 per cent SDS in water. The samples were finally mixed with an equal volume of twice concentrated "final sample buffer" and boiled for 1 min before electrophoresis. The sampling time is indicated at the bottom of the gels. All the preliminary experiments were done with wild type phage, but this experiment was performed with the double mutant in genes 10 and 18 in view of plans for future experiments.

Evidence for the precursor–product conversion IP→IP* is provided by the observation that the disappearance of IP and the appearance of IP* is coordinated in time (Fig. 5*b*). Furthermore, the total label lost at the IP position is recovered in the IP* band. The reaction is slower than that of P23 and P22. Only about 50 per cent of the total label at the IP position disappears. This could be explained either by another protein band overlapping with the IP band or synthesis in excess.

IP* cannot arise from P22. IP* must be derived from a precursor which is synthesized early, for I have shown (Fig. 1) that IP* is strongly labelled in the "early labelled" phage preparation. P22, however, is reported to be synthesized at late times only[12], which I have confirmed. The precursor conversion of IP→IP* has also been observed in a pulse-chase experiment in which the label was added between 4 and 5 min following infection, thus labelling only early proteins (results not shown). The band IP was easily recognized in these gels and the disappearance of IP and the appearance of IP* were again correlated in time. This quantitative agreement and the absence of other unaccounted changing bands in the gel pattern support the argument for the IP→IP* relationship. Of course, a final proof awaits chemical analysis.

It was also observed that, although the pulse was performed between 4 and 5 min after infection, cleavage of IP starts only

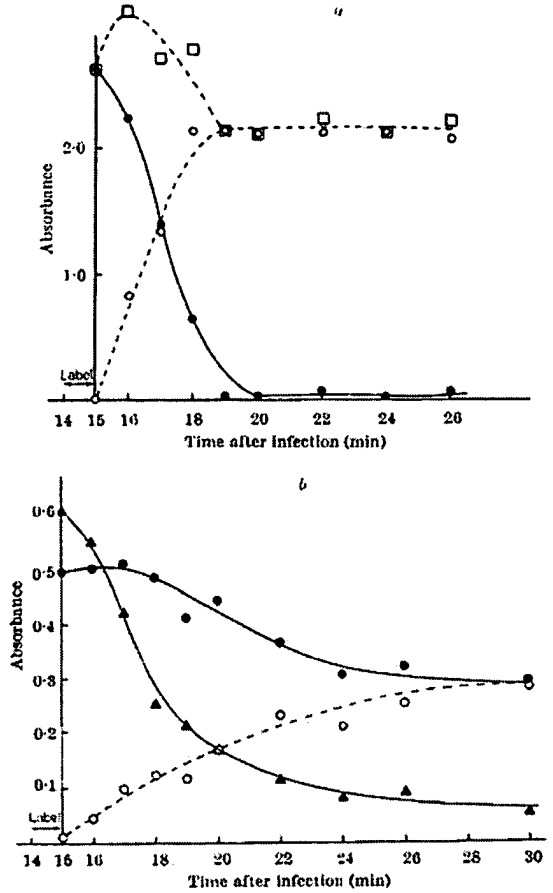

Fig. 5. Kinetics of cleavage of P23, P22 and IP. The kinetics of cleavage were measured using a microdensitometer (double beam recording microdensitometer, Joyce-Loebl) to record the autoradiogram. The exposure of the autoradiogram was chosen so that the absorbance of the band to be measured did not exceed 1 unit. The abscissa represents the integrated absorbance over the relevant peaks. *a*: ●—●, integrated absorbance over the P23 peak; ○···○, integrated absorbance over the P23* peak; □···□, total absorbance in P23 and P23*. *b*: ▲—▲, integrated absorbance over peak P22; ●—●, integrated absorbance over peak IP; ○···○, integrated absorbance over peak IP*.

at late times (after 17 min). Phage assembly starts at about this time and it is therefore thought that the cleavage of IP is linked to phage assembly.

Cleavage of P24. P24* (mol. wt 43,500) appears coordinately with P23* and P22* (Fig. 4) (the kinetics are not plotted). The precursor product relationship P24→P24* is more difficult to demonstrate, because P24 migrates only slightly faster than P23*, but the following experiment proves that P24 is missing in a pulse-chase wild type lysate. P24 separates somewhat better from P23* in a gel of lower concentration. The samples of the pulse-chase experiment were analysed on 8 per cent acrylamide gels and four time points are presented in Fig. 6. P24 is easily distinguished from the small amount of P23* existing immediately after the chase of the radioactive label (Fig. 6, 15 min). P24 disappears at the same time as P24* appears while P23* increases. One might argue that P24 is obscured by the heavy P23* band. This possibility was excluded by adding a lysate (23-defective lysate) containing P24 to the final samples of the pulse-chase experiment. P24 was then detected and it can be concluded that measurements of P24 are reliable, thus showing that P24 most likely gives rise to P24*. The integrated absorbance values over these two bands are about equal, but the small loss of protein weight by the P24→P24* reaction is not likely to be detected by the densitometric measurements. P24 could not give rise to IP*, because the total label in IP* is two or three times larger than that of P24 and P24 is synthesized late. Moreover, the precursor relationship of P24→P24* is considerably strengthened by recent observations on head maturation genes (my unpublished results). P24 does not seem to be cleaved at all in 50-defective cells and, indeed, no P24* is found. Cleavage of P23, P22 and IP does occur in 50-defective cells, although at a reduced rate.

Fate of the Small Fragments

Where are the small fragments of these cleavage reactions? The expected molecular weights for the small fragments stemming from P23, IP and P24 would be about 10,000, 2,500 and 1,500 respectively. Peptides of this size are not sieved on 10 per cent acrylamide gels and migrate with the marker dye (unpublished results of U. K. L. and J. V. Maizel). Attempts to find at least the 10,000 molecular weight fragment from P23 on gels of higher acrylamide concentration have failed. Possibly the fragments are further broken down to undetectable sizes. Fragments of P22 also have not been detected. Acid-soluble components which are derived from an acid-insoluble precursor are known to exist in T4 infected cells[17]. Two are associated with the phage particle and they are released with the DNA from the head upon osmotic shock[17]. The genetic determinant of one of these internal peptides has been mapped recently and lies in the neighbourhood of genes 20 and 21 (ref. 18). My results definitely rule out gene 20, which is incorporated unmodified into normal phage. Unfortunately, I have not discovered the product of gene 21 in the gel pattern. These results do not rule out the possibility that one of the internal proteins is derived from the small cleavage fragment of P22, P23 or P24. The appearance of the internal peptides seems indeed to be coordinated with the cleavage reactions of P23, P22 and P24. Genes 20, 21, 22, 23, 24 and 31, which affect the cleavage of P23, P22 and IP, are known also to affect the appearance of the internal peptides[17].

It has been pointed out to me by S. Bronner and A. Stretton that the cleavage point must occur at the N-terminal end of the P23 protein. In establishing the co-linearity of gene 23 and its polypeptide[1], they observed peptides in 23-defective lysates which contain the amber fragments, but these are absent in wild type lysates or in purified phage particles. These peptides may be derived from the N-terminal end of the amber fragment, which is cleaved off in the protein P23*. These observations also suggest that the small cleavage fragment, with an expected molecular weight of about 10,000, is fragmented to even smaller pieces.

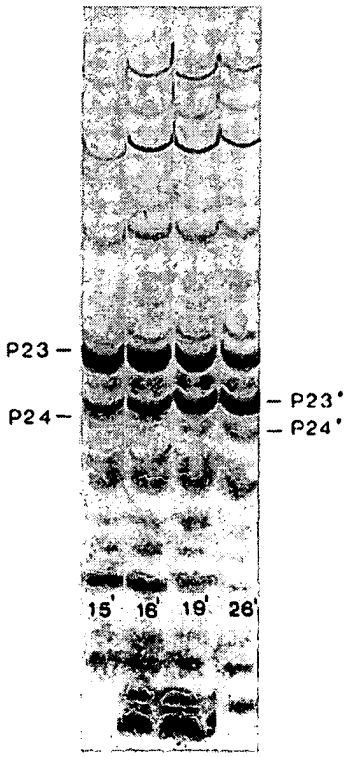

Fig. 6. Cleavage of the product of gene 24 (8 per cent acrylamide gels). Some samples (15, 16, 19 and 26 min) of the pulse chase experiment of Fig. 4 were analysed on 8 per cent acrylamide gels. Only the relevant part of the gel pattern is shown.

Cleavage occurs in a Large Structure

The observation that all the proteins P20, P21, P22, P29, P24 and P31 are required for efficient cleavage of P23, P22, P24 and IP suggests that these precursor proteins aggregate first to form an oligomeric structure, and are cleaved subsequently, rather than being cleaved first, and then assembled. The following experiment supports this view. The experiment is based on the observation that most of the precursor proteins are soluble and monomeric in SDS at room temperature, but that phage particles are not totally disrupted, as is also true in urea[12]. The gel pattern of purified phage treated with SDS at room temperature is compared with completely degraded phage, boiled for 1 min in SDS (Fig. 7*a* and *b*). Only a small fraction of P23* is extracted from phage with SDS at room temperature. Most of the proteins stay at the top of the gel or enter the gel as high molecular weight aggregates. Some proteins are not extracted at all. Note, however, that a few proteins are almost quantitatively extracted from the phage particles. The samples from the pulse-chase experiment treated in SDS at room temperature only are shown in Fig. 7. P23 disappears with time but no P23* appears, suggesting that P23 enters an SDS-resistant structure before being cleaved. Of course, these experiments cannot rule out the possibility that P23* is converted to an SDS-resistant structure so rapidly that it is not detected. A high molecular weight protein appeared at the top of the gel, which could be an aggregate of P23 but only accounts for part of the label which disappears from P23. Most of the label stays at the top of the gel. The molecular weight of this structure must therefore be greater than 300,000, for such a molecular weight is excluded from these gels (unpublished results of U. K. L. and J. V. Maizel). It is possible that this structure has a capsid-like shape. IP* and P24* are not resolved in these gels. This is because of the high salt concentration in these samples ('M9' growth medium) which impairs the resolution of the gel in the low molecular weight region.

Maturation of the Head

My experiments demonstrate that the assembly of the head of bacteriophage T4 is not a simple, straightforward self-assembly, because several structural proteins are chemically altered at some stage of assembly. The uncleaved precursor protein P23 can, however, be polymerized into single and multilayered polyheads and τ-particles, if its cleavage is blocked as a result of mutation.

Investigations of genes 2, 4, 13, 14, 16, 17, 49, 50, 64 and 65, which supposedly control late steps in head formation, are in progress. It is interesting that the cleavage of P23, P22, P24 and IP seems to be normal in cells infected with phage carrying mutations in these genes with the exception of genes 2, 50 and 64 (my unpublished results).

Why are these structural head components cleaved? The finding that IP gives rise to an internal IP* sheds light on a possible consequence of the cleavage reactions. Internal proteins are thought to bind to DNA and the cleavage reactions may possibly trigger the necessary DNA–protein interactions, which result in orderly packing of the DNA within the shell of proteins. The following model may be proposed. The proteins P20, P21, P23, P24, P31 and IP form an intermediate structure (SDS-resistant) which combines with an end of a DNA strand. Cleavage of P23, P22, P24 and IP proceeds from the DNA attachment site. During this process more and more DNA binding sites may be formed at the inside of this structure, perhaps by the formation of IP*, thus winding up the DNA strand successively. The small acid-soluble peptides formed during this process may also interact with the packed DNA[17] to neutralize charges. My results do not decide whether DNA packing proceeds simultaneously with the polymerization of the head membrane or follows thereafter.

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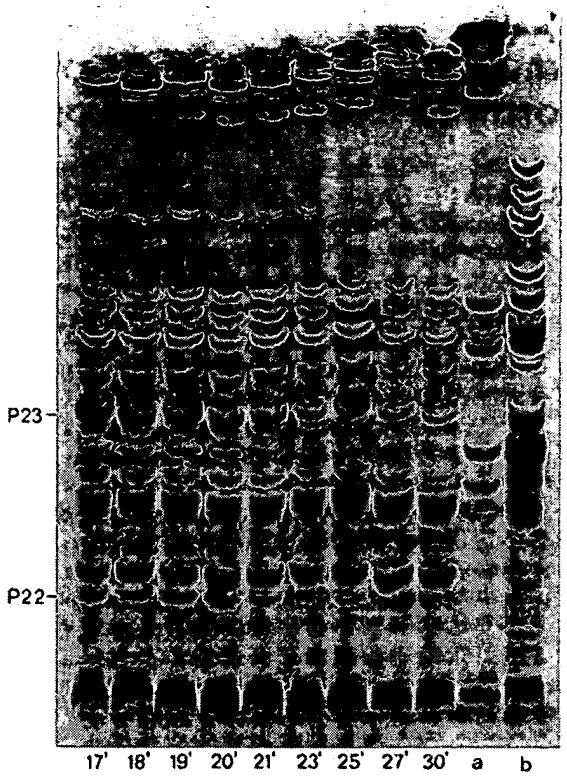

Fig. 7. Chase of P23 into a product stable to SDS at room temperature. A culture was infected and pulse labelled as described in Figs. 1 and 3 with the following changes. The culture was grown at 30° C, infected with wild phage, and labelled for 2 min from 15–17 min after infection. The samples were mixed at room temperature with an equal volume of twice concentrated "final sample buffer" without previous dialysis. The samples were then directly applied to the gels without being boiled. The sampling time is indicated at the bottom of the gels. As a control a purified phage preparation is analysed. *a*, Phage treated in SDS at room temperature only; *b*, phage boiled in SDS for 1-5 min.

Note added in proof. During the preparation of this manuscript I was informed that the alteration of P23 has also been observed by other workers: E. Kellenberger and C. Kellenberger-van der Kamp, *FEBS Lett.*, **8**, 3, 140 (1970); R. C. Dickson, S. L. Barnes and F. A. Eiserling, *J. Mol. Biol.* (in the press); and J. Hosoda and R. Cone, *Proc. US Nat. Acad. Sci.* (in the press).

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